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Selenocysteine mimics the effect of dietary restriction on lifespan via SKN-1 and retards age-associated pathophysiological changes in *Caenorhabditis elegans*

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Abstract. Selenocysteine, a sulfur-containing amino acid, can modulate cellular oxidative stress defense systems by incorporating into anti-oxidant enzymes such as glutathione peroxidase and thioredoxin reductase. Selenocysteine can also prevent cancer, neurodegenerative diseases and cardiovascular diseases. A recent study revealed that dietary supplementation with selenocysteine can increase the resistance of *Caenorhabditis elegans* to environmental stressors and its lifespan. The objective of the present study was to identify the underlying mechanism involved in the lifespan-extending effect of selenocysteine and the effect of selenocysteine on age-associated pathophysiological changes. Lifespan assays with known long-lived mutants of *age-1* (the ortholog of the phosphoinositide 3-kinase), *clk-1* (the ortholog of demethoxyubiquinone hydroxylase) and *eat-2* (a ligand-gated ion channel subunit) revealed that the effect of selenocysteine on lifespan specifically overlapped with that of the *eat-2* mutation, a genetic model of dietary restriction (DR). Selenocysteine mimicked the effect of DR on the bacterial dilution method. It required SKN-1 (the ortholog of mammalian nuclear factor-erythroid-related factor) for lifespan extension. In addition, selenocysteine significantly delayed the paralysis induced by human amyloid-β gene, positively correlated with the incidence of Alzheimer's disease. The effect of selenocysteine on amyloid-β-induced toxicity was dependent on the nuclear localization of DAF-16. Reduced survival caused by high-glucose-diet was recovered by selenocysteine. Selenocysteine also reduced the cellular level of reactive oxygen species known to be increased by high-glucose-diet. The results of the present study suggested that selenocysteine can mimic the effect of DR on lifespan and age-associated pathophysiological alterations, providing scientific evidence for the development of DR mimetics using selenocysteine.

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Introduction

A lot of studies have been performed to understand the aging process and identify causal factors of aging. However, the exact mechanism of aging has not been fully elucidated yet. Other major research areas in aging are focused on finding ways to slow down the aging process and retard age-related pathophysiological changes. Among many genetic and environmental interventions obtained from model organisms so far, the only intervention that shows consistent lifespan extensions with positive effects on health span in all experimental organisms tested is dietary restriction (DR). The first report about the effect of DR effect on aging was done with rats in 1917 (1). Dietary-restricted rats showed increased lifespan and extended reproductive period compared to not-restricted rats. Since then, the effect of DR has been replicated in many organisms ranging from yeast to monkeys. In *Drosophila melanogaster*, DR using dilution of yeast or sugar has increased lifespan, imposing a reduced reproduction as a trade-off (2). Mice fed with 40% reduced calories have shown lifespan extension with reduced incidence and/or delayed onset of age-related pathologies (3). A recent study with rhesus monkeys has revealed that DR could significantly extend lifespan and delay the onset of many age-related pathophysiological changes, including incidence of cancer, diabetes mellitus, and brain atrophy (4). There are many different methods of DR in *C. elegans*. Dilution of bacterial culture, a common food source for *C. elegans* growth, can lead to lifespan extension (5). Mutations in *eat-2* gene can lead to reduced pharyngeal food pumping rate and confer a longevity phenotype (6). Worms grown in liquid synthetic medium containing no bacteria also have an increased lifespan (7).

Despite conserved effect of DR on various organisms, it is impracticable for humans because it requires long-term restraint. The anti-aging effect of DR completely disappears within a few days when dietary-restricted

Drosophila melanogaster are fed *ad libitum* (AL) (8). Therefore, people have attempted to discover DR mimetics that can lead to DR response without food restriction. Resveratrol, a polyphenol compound found in red wine, has been shown to induce lifespan extension via activation of sirtuin which is required for DR response (9). Genomic transcriptional profiling has revealed that dietary supplementation with resveratrol causes similar transcriptional changes observed with long-term DR (10). Resveratrol also has preventive effect against age-related neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (11). Metformin, a drug prescribed for treatment of type 2 diabetes, can extend lifespan and reduce the incidence of age-related diseases such as cancer, chronic kidney disease, and cardiovascular diseases in mice (12-14). The lifespan extension effect conferred by metformin is dependent on the activation of AMP-activated protein kinase (AMPK) known to modulates DR response in *C. elegans* (15,16). In addition, the effect of metformin on transcriptional profile overlaps with the effect of DR in mice (17). A recent study has reported that D-allulose, an isomer of D-fructose, can increase lifespan and mimic DR response in *C. elegans* (18). The lifespan-extending effect of D-allulose does not accompany a reduced food intake or require AMPK (18). Further studies focusing on underlying mechanisms of DR mimetics and the effect of DR mimetics on age-related pathophysiological changes are necessary to identify relevant DR mimetics.

Selenocysteine is a cysteine derivative in which selenium substitutes sulfur in the side chain of cysteine. Selenocysteine is incorporated into selenoproteins known to modulate anti-oxidant defense and cellular redox regulation (19). Lack of selenocysteine is associated with a number of age-related diseases, including cancer, cardiovascular diseases, and neurodegenerative diseases (20-22). Selenocysteine-containing peptides show protective effects against hepatic ischemia-reperfusion injury by decreasing free radicals and apoptosis in rats (23). Intraperitoneal injection of selenium can reduce oxidative toxicity induced by scopolamine and confer neuroprotection in hippocampus of rats (24). Mutations in selenocysteine synthase gene encoding an enzyme required for selenocysteine biosynthesis can lead to congenital cerebellar atrophy in humans (25). In *C. elegans*, organochalcogen containing selenium can inhibit Mn-induced toxicity and induce increased expression of superoxide dismutase-3 and nuclear localization of DAF-16 (26). Selenoprotein can also reverse age-related decline of molting in *C. elegans* (27). Our previous study has shown that dietary supplementation with selenocysteine can increase resistance to environmental stressors such as oxidative stress and ultraviolet irradiation in *C. elegans* (28). Selenocysteine can also extend lifespan of *C. elegans* without reducing its fertility. It can also delay age-related decline of its motility (28).

In the present study, we investigated the underlying mechanism involved in the lifespan-extending effect of selenocysteine using known long-lived genetic mutants of *C. elegans*. The effect of selenocysteine on age-related pathophysiological changes was then elucidated employing genetic or nutritional interventions. Results of this study will broaden our understanding of biological activities of selenocysteine and its possible application to human health.

Materials and methods

Worm strains and maintenance. Wild-type N2 strain and all mutant strains were purchased from *C. elegans* Genetics Center (CGC, Minneapolis/St. Paul, MN, USA). Long-lived mutants *age-1* (*hx546*), *clk-1* (*e2519*), and *eat-2* (*ad465*) were used to identify underlying mechanism for the lifespan-extending effect of selenocysteine. CL4176 strain, the genetic disease model of AD, contains human amyloid-β $(A\beta)_{1-42}$ [dvls27 (*myo*-3/$A\beta_{1-42}$/*let* UTR, *rol-6*)] transgene inducible in muscle tissues. TJ356 strain carries a transgene *daf-16* fused to GFP [zls356 IV (*daf-16p::daf-16a/b::*GFP, *rol-6*)]. Worms were maintained on Nematode Growth Media (NGM) plates (1.7% agar, 2.5 mg/ml peptone, 25 mM NaCl, 50 mM KH_2PO_4 pH 6.0, 5 μg/ml cholesterol, 1 mM $CaCl_2$, and 1 mM $MgSO_4$) spotted with *Escherichia coli* OP50 as food source.

Lifespan assay. Age-synchronization was accomplished by letting five young adult worms lay eggs on a fresh NGM plate for 4 h at 20˚C. These eggs were then incubated at 20˚C for 3 days. Among age-synchronized young adult worms, 60 worms were randomly selected for each group and transferred to fresh NGM plates. To inhibit internal hatching, 5-fluoro-2'-deoxyruridine (12.5 mg/l) was added to NGM plates. The number of worms alive, dead, or censored was counted daily. Censored worms included killed, lost, or bagged worms during assays. They were excluded from the statistical analysis.

DR on solid NGM plates. DR was performed using bacterial dilution method. After growing *E. coli* OP50 at 37˚C, bacteria were prepared at density of $5x10^9$ bacteria/ml for AL group and $5x10^8$ bacteria/ml for DR group using serial dilution. Then 200 μl of each bacteria culture was spotted onto solid NGM plates containing 5-fluoro-2'-deoxyuridine and ampicillin. The survival of age-synchronized worms was monitored daily until all worms were dead following the lifespan assay described previously.

RNA interference (RNAi). E. coli clones harboring each gene for RNAi were obtained from Ahringer RNAi library (29). Their sequences were verified. The expression of double-stranded RNA was induced by 0.4 mM isopropyl-β-D-thio-galactoside (IPTG; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 4 h after OD_{600} reached 0.4. Cultured medium was spotted onto NGM plates containing 100 μg/ml ampicillin, 12.5 μg/ml tetracycline, 0.4 mM IPTG, and 0.5 mg/ml 5-fluoro-2'-deoxyuridine. The bacterial clone containing empty vector (EV) was used as a negative control.

Aβ-induced toxicity assay. Five age-synchronized young adult CL4176 worms were left to lay eggs for 5 h at 20˚C. After removing five adult worms from the plate, eggs were incubated for 5 d at 15˚C. Thirty young adult worms were then randomly selected and allowed to lay eggs for 2 h at 15˚C. Their progeny were grown at 25˚C for 24 h. Percentage of paralyzed worms by the induction of Aβ in muscle was then recorded (n=60).

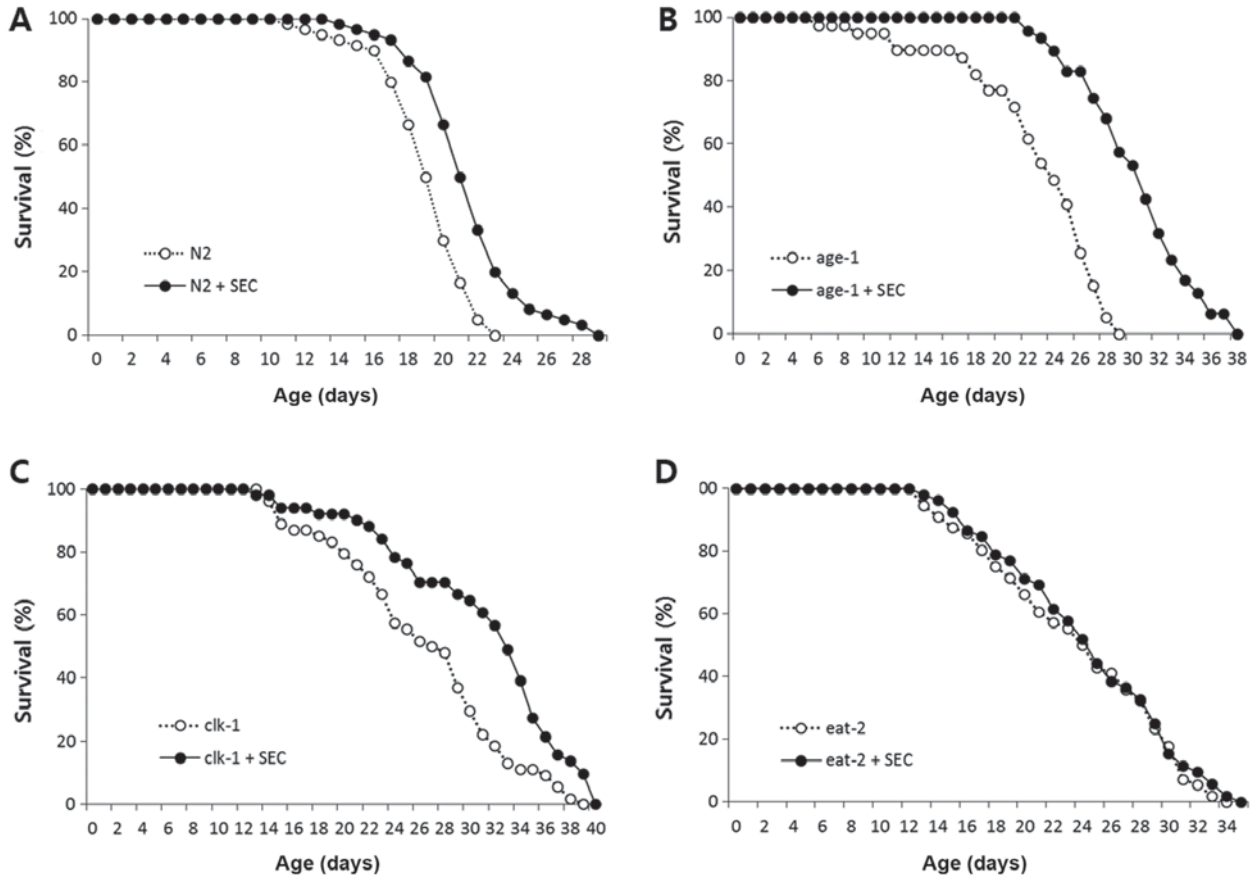

Figure 1. Effect of selenocysteine specifically overlaps that of *eat-2*. Comparisons of the lifespans of (A) wild-type N2, (B) *age-1*, (C) *clk-1* and (D) *eat-2* between the untreated control and selenocysteine-treated groups (n=60). Selenocysteine significantly increased the lifespan of N2, *age-1* and *clk-1* ($P<0.05$), but not that of *eat-2*. SEC, 5 mM selenocysteine; *clk-1*, demethoxyubiquinone hydroxylase ortholog; *age-1*, phosphoinositide 3-kinase ortholog; *eat-2*, a ligand-gated ion channel subunit.

Subcellular distribution of DAF-16. Age-synchronized TJ356 worms were supplemented with 5 mM selenocysteine for 9 days at 20˚C. Each worm (n=60) was anaesthetized with 1 M sodium azide on a slide glass coated with 2% agarose. Subcellular localization of DAF-16::GFP was then monitored with a confocal microscope (Olympus FV10i; Olympus Corporation, Tokyo, Japan). Each worm was distributed into three classes according to subcellular localization of DAF-16::GFP: Cytosolic, nucleus, or intermediate (both cytosolic and nuclear).

High-glucose-induced toxicity assay. Randomly selected 60 age-synchronized worms were transferred to a fresh NGM plate containing glucose (40 mM). The survival of worms in untreated control group was compared to that of glucose-treated group. The effect of 5 mM selenocysteine on high-glucose-induced toxicity was measured at 20˚C.

Determination of cellular reactive oxygen species (ROS). Three-day-old age-synchronized worms were treated with or without 5 mM selenocysteine for 7 days at 20˚C. Individual worm was then transferred to a 96-well black plate containing 190 μl of PBST per well (n=20). After adding 10 μl of H_2DCF-DA (Sigma-Aldrich), fluorescence intensity of each animal was determined using a fluorescence multi-reader (Infinite F200; Tecan, Grodig, Austria).

Statistical analysis. For the lifespan assay and toxicity assay, we used the log-rank test (30). The log-rank test is a non-parametric Mantel-Cox test used for statistical comparison between two survival curves. For subcellular distribution of DAF-16 and cellular ROS levels, we employed the standard two-tailed Student's t-test. Statistical analyses were performed using Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Overlapping effect of selenocysteine and eat-2 mutation on lifespan. After observing lifespan-extending effect of selenocysteine in our previous study, we investigated the underlying mechanisms involved in the longevity phenotype conferred by selenocysteine in the present study (28). Three well-known long-lived mutants representing different lifespan-extending pathways were employed. Mutant *age-1* has an increased lifespan due to reduced insulin/IGF-1-like signaling (31). Mutation in *clk-1* produces less ROS due to reduced mitochondrial electron transport chain reaction that can lead to lifespan extension (32). Mutant *eat-2* is a genetic model of DR. It intakes less food due to reduced pharyngeal pumping rate (6). As previously reported, dietary supplementation with selenocysteine increased the lifespan of wild-type control

Table I. Effect of selenocysteine on the lifespan of the wild-type N2 strain and long-lived mutants.

Group	Experiment no.	Mean lifespan (day)		P-value
		Control	Selenocysteine (5 mM)	
N2	1st experiment	21.0	29.8	0.004
	2nd experiment	20.2	21.6	0.027
age-1	1st experiment	22.7	30.4	<0.001
	2nd experiment	24.2	28.9	0.016
clk-1	1st experiment	26.4	31.1	0.001
	2nd experiment	16.6	19.8	0.009
eat-2	1st experiment	23.8	24.5	0.557
	2nd experiment	18.6	19.1	0.728

clk-1, demethoxyubiquinone hydroxylase ortholog; *age-1*, phosphoinositide 3-kinase ortholog; *eat-2*, a ligand-gated ion channel subunit.

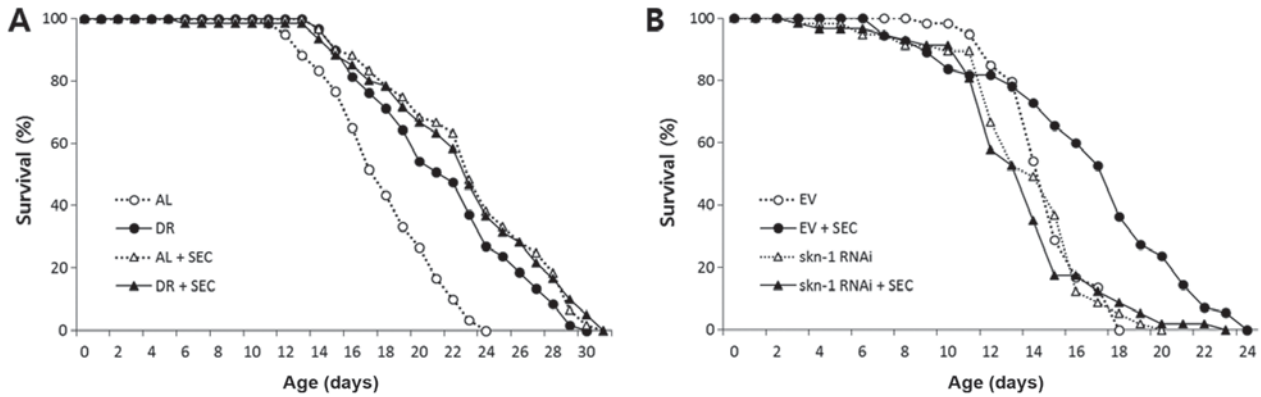

Figure 2. Selenocysteine requires SKN-1 to mimic DR. (A) The effect of bacterial dilution and supplementation with selenocysteine on lifespan was determined (n=60). There was no additional increase in lifespan when diluted bacteria and selenocyteine were simultaneously applied. (B) The increased lifespan by selenocysteine was completely abolished by *skn-1* knockdown using RNAi. AL, *ad libitum* ($5x10^9$ bacteria/ml); DR, dietary restriction ($5x10^8$ bacteria/ml); SEC, 5 mM selenocysteine; RNAi, RNA interference; EV, empty vector RNAi clone; SKN-1/*skn-1*, mammalian nuclear factor-erythroid-related factor ortholog.

N2 (Fig. 1A). Selenocysteine also significantly extended lifespans of long-lived *age-1* and *clk-1* (Fig. 1B and C). The mean lifespan of *age-1* was 22.7 d and that of *age-1* treated with 5 mM selenocysteine was 30.4 d (P<0.001). The mean lifespan of *clk-1* was increased from 26.4 d in the untreated control to 31.1 d in the selenocysteine-treated group (P<0.001). However, there was no significant difference in the lifespan between untreated control and selenocysteine-treated *eat-2* mutants (Fig. 1D). Independent replicative experiment also showed similar results (Table I). Results of these experiments indicate that the effect of selenocystein on lifespan specifically overlaps with that of *eat-2* mutation, a genetic model of DR.

Selenocysteine mimics DR and requires SKN-1 for lifespan extension. To confirm the overlapping effect of selenocysteine and DR on lifespan, we examined the effect of selenocysteine on lifespan of dietary-restricted worms. Compared to AL worms whose mean lifespan was 17.9 days, the mean lifespan of dietary-restricted worms was increased to 21.5 days (P<0.001; Fig. 2A). Supplementation with selenocysteine also significantly extended its lifespan (mean lifespan: 22.6 days). However, simultaneous intervention with DR and selenocysteine failed to further increase lifespan. The mean lifespan of worms treated with both DR and selenocysteine was 20.7 days, which was not significantly different from the mean lifespan of worms treated with either DR or selenocysteine (Fig. 2A and Table II). Next, we determined whether lifespan extended by selenocysteine was mediated by the same factor that modulated DR response. SKN-1 is a transcription factor known to regulate responses to oxidative stress and DR-induced lifespan extension in *C. elegans* (33). RNAi of *skn-1* induces cell fate transformation during development and modulates oxidative stress response in adult worms (34,35). Interestingly, the extended lifespan conferred by selenocysteine was completely blocked by gene knockdown of *skn-1* using RNAi (Fig. 2B). Mean lifespans of worms treated with and without selenocysteine were 16.7 and 14.7 days, respectively (P<0.001). The mean lifespan of worms treated with both *skn-1* RNAi and selenocysteine (13.5 days) was not significantly different from that of worms treated with *skn-1* RNAi alone (13.8 days) (Table III). Another well-known DR-mediating factor is DAF-16, a FOXO transcription factor that can modulate stress response. Gene knockdown of *daf-16* by RNAi reduces resistance to

Table II. Effect of selenocysteine and dietary restrictions on the lifespan of the wild-type N2 strain.

A, First experiment

Group	Mean lifespan (day)	P-value
AL	17.9	
DR	21.5	<0.001[a]
AL+SEC	22.6	<0.001[a]
DR+SEC	20.7	0.514[b]

B, Second experiment

Group	Mean lifespan (day)	P-value
AL	14.6	
DR	17.1	<0.001[a]
AL+SEC	16.7	<0.001[a]
DR+SEC	18.1	0.171[b]

[a]Comparisons vs. AL survival; [b]Comparisons vs. DR survival. AL, *ad libitum* ($5x10^9$ bacteria/ml); DR, dietary restriction ($5x10^8$ bacteria/ml); SEC, 5 mM selenocysteine.

oxidative stress and decreases lifespan in *C. elegans* (36,37) However, knockdown of *daf-16* did not significantly affect the lifespan-extending effect of selenocysteine (Table III). These findings validate the effect of selenocysteine as a DR mimetic, suggesting that lifespan extension by selenocysteine requires SKN-1. However, it is not dependent on DAF-16.

Selenocysteine decreases Aβ-induced toxicity through DAF-16. Previous studies have shown that DR can retard many age-related pathophysiological changes (38). We tested the effect of selenocysteine on Aβ-induced toxicity using genetic AD animal model. Induction of transgenic human Aβ gene in muscle tissues caused paralysis in *C. elegans* (mean survival time 7.0 h). However, dietary supplementation with selenocysteine markedly suppressed the rate of paralysis (Fig. 3A). Mean survival time was increased to 9.4 h, which was increased by 34% when compared to that of untreated control (P<0.001; Table II). Next, we examined the effect of *daf-16* knockdown on Aβ-induced paralysis. DAF-16 is known to be required for protection against Aβ toxicity (39). Surprisingly, the inhibitory effect of selenocysteine on Aβ-induced paralysis disappeared when the expression of *daf-16* was specifically knocked down by RNAi (Fig. 2B). Mean survival time was not significantly different between EV control group (7.2 h) and *daf-16* RNAi group (7.9 h). Supplementation with selenocysteine failed to increase mean survival time without DAF-16 (7.8 h) (Table IV). Based on previous finding that the lifespan-extending effect of selenocystein required SKN-1, we also examined the effect of *skn-1* knockdown on Aβ-induced paralysis. Unlike results obtained with *daf-16* RNAi, selenocysteine significantly increased survival time after Aβ induction with *skn-1* RNAi (Table IV). Our data showed that the lifespan-extending effect of selenocysteine could retard pathophysiological changes in AD animal model. In addition, DAF-16, but not SKN-1, was necessary for the preventive effect of selenocysteine against Aβ-induced toxicity in *C. elegans*.

Selenocysteine induces nuclear localization of DAF-16. DAF-16 is transferred to nucleus in response to environmental stressors such as oxidative stress, heat shock, and UV irradiation (40). DR also induces nuclear localization of DAF-16 (40,41). Based on the result that DAF-16 was required for increased resistance to Aβ-induced toxicity by selenocysteine, we hypothesized that selenocysteine might induce nuclear localization of DAF-16. Using DAF-16::GFP transgene, we classified subcellular distribution of DAF-16 into three categories: Cytosolic, intermediate, and nucleus (Fig. 4A). Dietary supplementation with selenocysteine induced nuclear localization of DAF-16 without any environmental stimuli. In the untreated control group, 56.1±2.00% (mean ± SEM) of worms showed cytosolic distribution of DAF-16 while only 17.2±7.78% of worms showed cytosolic distribution of DAF-16 in selenocysteine-treated worms (P=0.008). The percent of intermediate distribution which showed both cytosolic and nucleus localization of DAF-16 was increased from 41.1±0.012% in the untreated control group to 74.5±6.96% in the group with supplementation of selenocysteine (P=0.012). In addition, higher number of animals showed nuclear localization of DAF-16 in the selenocysteine-treated group compared to that in the untreated control group. Percent of nuclear distribution was 2.8±1.47% in the untreated control group and 8.3±0.96% in selenocysteine-treated group (P=0.034; Fig. 4B). Therefore, selenocysteine could confer resistance to Aβ-induced toxicity through induction of nuclear localization of DAF-16 in *C. elegans*.

Selenocysteine reduces high-glucose-induced toxicity and cellular ROS. DR can improve glucose homeostasis and ameliorates diabetes mellitus (42). Metformin, a widely-used medicine for type 2 diabetes, can mimic DR responses in *C. elegans* (16). We thus determined whether DR-mimicking selenocysteine could reduce high-glucose-induced toxicity. As shown in Fig. 5A, high glucose increased mortality of *C. elegans*. Mean lifespan was decreased from 16.4 days in the untreated control group to 12.4 days in the high-glucose-treated group, which was a decrease of 24% (P<0.001). However, the reduced lifespan due to high-glucose-toxicity was completely recovered when selenocysteine was simultaneously treated with high glucose. Triple replicative experiments also showed similar results (Table V). A previous study has shown that high-glucose-diet can induce an increase in ROS level while chicoric acid, an anti-diabetic molecule, can increase lifespan and reduce cellular ROS level (43). We then measured cellular ROS levels in the untreated control group and selenocysteine-treated group. Fluorescence intensity representing cellular ROS level was significantly decreased after supplementation with selenocysteine (Fig. 5B). After 1 h of incubation with H_2DCF-DA, relative fluorescence intensity was decreased to 64±5.7% (mean ± SEM) in the group with supplementation of selenocysteinein compared to that of the untreated control group (100±16.8%, P=0.047). There was also a significant decrease in relative fluorescence intensity by supplementation with selenocysteine after 2 h of

Table III. Effect of *skn-1* or *daf-16* RNAi on the lifespan extension induced by selenocysteine.

Group	Experiment no.	Mean lifespan (days)		P-value
		Control	Selenocysteine (5 mM)	
EV	1st experiment	14.7	16.7	<0.001
	2nd experiment	13.7	16.7	<0.001
skn-1 RNAi	1st experiment	13.8	13.5	0.633
	2nd experiment	21.4	18.9	0.254
daf-16 RNAi	1st experiment	12.5	15.6	<0.001
	2nd experiment	12.8	14.8	<0.001

EV, empty vector; *skn-1*, mammalian nuclear factor-erythroid-related factor ortholog; RNAi, RNA interference.

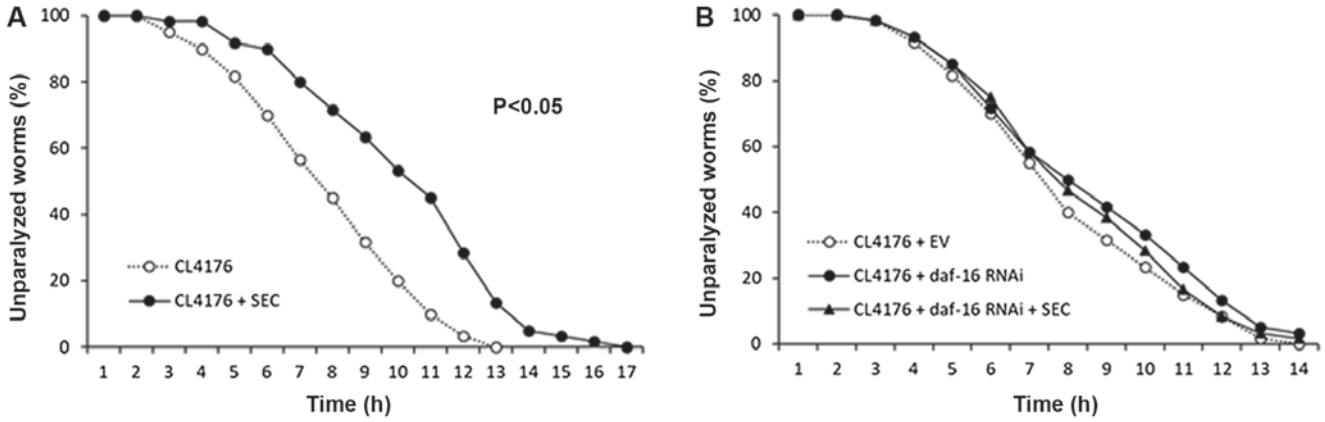

Figure 3. Reduced Aβ-induced toxicity via DAF-16. (A) The number of paralyzed worms by muscle-specific induction of human Aβ gene was monitored using the CL4176 strain (n=60). Selenocysteine significantly delayed paralysis caused by Aβ induction (P<0.05). (B) The requirement of DAF-16 for reduced Aβ-induced toxicity was examined using RNAi. When the expression of *daf-16* was inhibited, the effect of selenocysteine was completely blocked. The x-axis indicates the time after the induction of Aβ gene and the y-axis indicates the percentage of unparalyzed worms after the induction of the Aβ gene. All experiments were repeated twice. SEC, 5 mM selenocysteine; RNAi, RNA interference; EV, empty vector RNAi clone.

incubation with H_2DCF-DA [100±16.5 and 63±6.4% in the untreated control and selenocysteine-treated group, respectively (P=0.042)]. Independently repeated experiment also showed similar results. Relative fluorescence intensity was decreased to 67±6.8% (P=0.026) after 1 h of incubation with H_2DCF-DA and 66±6.6% (P=0.050) after 2 h of incubation with H_2DCF-DA. Taken together, these results suggest that selenocysteine can suppress high-glucose-induced toxicity by lowering cellular ROS levels.

Discussion

Positive impact of DR on lifespan and age-related diseases has been reported in many organisms. In human, centenarians live in Okinawa have less intake of calories compared to people live in other areas of Japan. They show lower incidence of cancer, Parkinson's disease, and AD (44). In the present study, the lifespan-extending effect of selenocysteine was not observed in *eat-2* mutant. However, lifespans of *age-1* mutant and *clk-1* mutant were significantly increased by selenocysteine. These findings suggest that the effect of selecocysteine on lifespan overlaps with that of DR. Previously, novel DR-specific genes have been identified using lifespan assay with the same three long-lived mutants. Genetic knockout of *nlp-7* or *cup-4* specifically abolished the longevity phenotype conferred by *eat-2* mutation, but not by *age-1* or *clk-1* mutation (45). In *C. elegans*, there are several methods of DR. Each DR method seems to involve independent and overlapping cellular signaling pathways. For example, *eat-2* mutation, a genetic model of DR, requires transcription factors SKN-1 and PHA-4. However, it is not dependent on DAF-16 for lifespan extension (46). The longevity phenotype caused by dilution of bacteria or peptone involves AMPK pathway and requires DAF-16 (15). Food deprivation extends lifespan via HSF-1 while lifespan extension by intermittent fasting is mediated by RHEB-1 in *C. elegans* (47,48). Results of the present study showed that selenocysteine mimicked the effect of DR through SKN-1 without requiring DAF-16. Additional studies are needed to determine the effect of selenocysteine on other methods of DR and identify downstream targets of selenocyteine for DR response. Such studies can broaden our understanding of cellular mechanisms involved in the effect of selenocysteine on lifespan.

Due to a difficulty of life-long restraint on food intake, people have searched DR mimetics that can induce DR response without restricting diet. Leading compounds that can

Table IV. Effect of *daf-16* or *skn-1* RNAi on reduced amyloid-β toxicity by selenocysteine.

Group	Experiment no.	Mean survival time (h)		P-value
		Control	Selenocysteine (5 mM)	
EV	1st experiment	7.0	9.4	<0.001
	2nd experiment	7.4	10.5	<0.001
daf-16 RNAi	1st experiment	7.9	7.8	0.836
	2nd experiment	7.6	7.9	0.461
skn-1 RNAi	1st experiment	7.0	9.2	0.017
	2nd experiment	8.6	10.6	0.015

EV, empty vector; *skn-1*, mammalian nuclear factor-erythroid-related factor ortholog; RNAi, RNA interference.

Table V. The effect of a high-gluocse diet and selenocysteine on the lifespan of the wild-type N2 strain.

Experiment	Mean lifespan (days)	P-value
1st experiment		
N2	16.4	
N2+Glucose	12.4	<0.001[a]
N2+Glucose+SEC	18.0	<0.001[b]
2nd experiment		
N2	17.0	
N2+Glucose	12.0	<0.001[a]
N2+Glucose+SEC	17.7	<0.001[b]
3rd experiment		
N2	15.0	
N2+Glucose	13.0	0.001[a]
N2+Glucose+SEC	16.0	<0.001[b]

[a]Comparison vs. N2. [b]Comparing vs. N2 + Glucose. Glucose, 40 mM glucose; SEC, 5 mM selenocysteine.

mimic DR are sirtuin activators. Sirtuin is a NAD-dependent histone deacetylase. Activation of SIRT1, a mammalian sirtuin, is known to be necessary for DR-induced long lifespan (48). Dietary supplementation with resveratrol increases both mean and maximum lifespans in yeast, nematode, and fruit fly. Such effect of resveratrol is dependent on sirtuin activation (9,49). Nicotinamide adenine dinucleotide (NAD^+, a cofactor of sirtuin) and oxaloacetic acid that can increase NAD^+ level are also strong candidates of DR mimetics that can modulate sirtuin activity (50). Mammalian target of rapamycin (mTOR) signaling that senses cellular nutrient status is known to be reduced by DR (51). Reduced mTOR signaling using genetic interventions or diet of rapamycin can increase lifespan in animal models (51). Rapamycin also has positive effects on age-related decline in cognitive function and cardiac function (52,53). Insulin/IGF-1-like signaling is one of most studied lifespan-modulating pathways conserved from invertebrates to vertebrates (54). Reduced plasma insulin observed in dietary-restricted animals is regarded as a biomarker of longevity (42). Metformin, a drug that can reduce plasma insulin level, can extend lifespan of *C. elegans* (16). Incidence of age-related diseases including cancer and cardiovascular disease can be markedly reduced by metformin (12,13). In mice, dietary supplementation with metformin can lead to genomic transcriptional profile resembling that of DR (17). A recent study has reported that dietary supplementation with N-acetyl-L-cysteine, a cysteine derivative with anti-oxidant activity, can mimic the effect of DR on lifespan and reduce Aβ-induced toxicity in *C. elegans* (55). Here, we demonstrated that the lifespan-extending effect of selenocysteine was achieved by mimicking DR response. S-allycysteine, another cysteine derivative, has also shown health span-promoting and lifespan-extending activities *in vivo* (56). Taken together, these results suggest that cysteine derivatives are a novel category of putative DR mimetics. The effect of cysteine derivatives including selenocysteine on aging process and onset of age-related diseases in higher organisms should be followed to evaluate cysteine derivatives as strong DR mimetics.

In addition to lifespan extension, DR can reduce incidence of many age-related diseases and retard various pathophysiological changes observed in aging mammals. DR has protective effects against AD, amyotropic lateral sclerosis, and various cancers (57,58). The progression of tumors can be delayed by DR in rodents while glucose intolerance and the severity of diabetes in mice can be improved by DR (34,42). A variety of age-related pathologies including cataracts, cognitive impairments, and muscle dysfunction can be attenuated by DR (38,59,60). A recent study of DR in rhesus monkeys has revealed that the incidence of cancer, type 2 diabetes, and cardiovascular disease is lower in DR animals compared to that in their counterparts fed with AL (4). The present study also showed that dietary supplementation of selenocysteine resulted in decreased susceptibility to Aβ-induced toxicity in AD genetic model. Such effect required DAF-6, but not SKN-1. Taken together, these results suggest that the increased lifespan and extended survival under Aβ-induced toxicity by supplementation with selenocysteine might be mediated by independent cellular pathways. Reduced survival due to high-glucose-diet was also completely recovered by supplementation with selenocysteine. These findings suggest that selenocysteine can ameliorate age-related pathophysiological changes and increase lifespan, achieving the same effect of

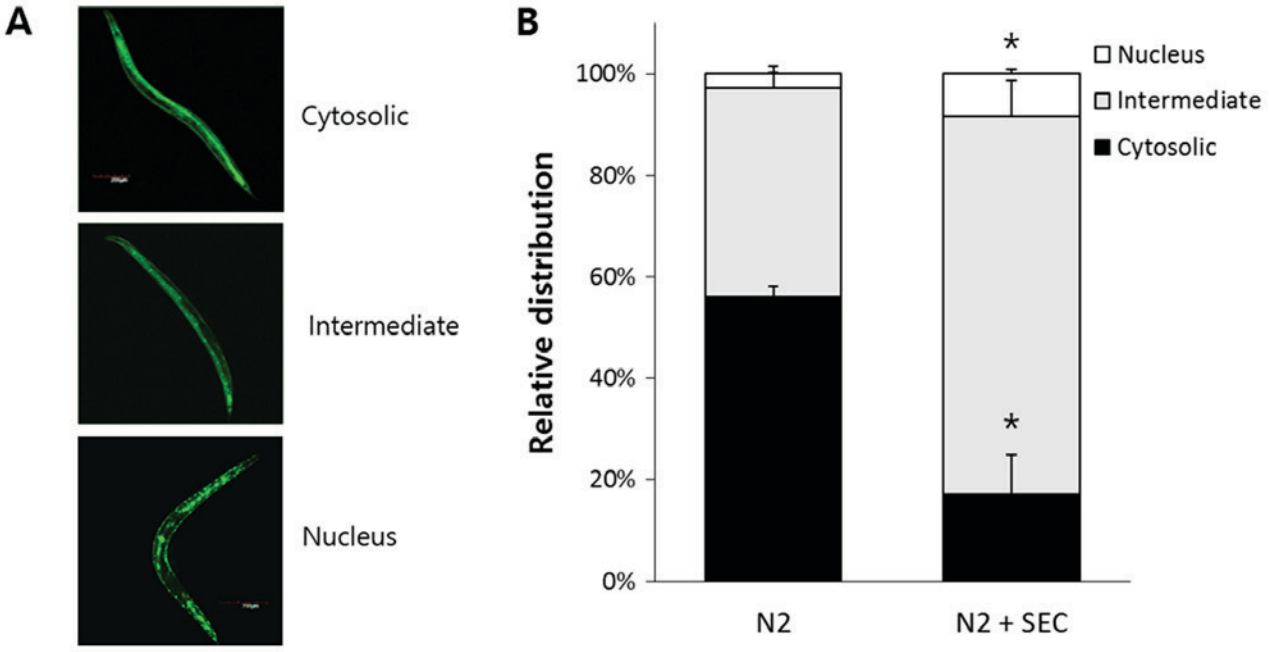

Figure 4. Changes in subcellular distribution of DAF-16 by selenocysteine. (A) A single worm exhibiting subcellular distribution of DAF-16::GFP is categorized into three groups: i) Cytosolic, a worm showing cytosolic expression of DAF-16::GFP; ii) intermediate, the expression of DAF-16::GFP in the cytosol and nucleus; and iii) nucleus, nuclear accumulation of DAF-16::GFP. (B) Relative distribution of each of the categories was compared between the untreated N2 group and the selenocysteine-treated group (n=60). More animals were categorized as 'Intermediate' and 'Nucleus' in the selenocysteine-treated group. Data is presented as the mean ± standard error of the mean. *P<0.05 vs. N2. SEC, 5 mM selenocysteine; GFP, green fluorescent protein.

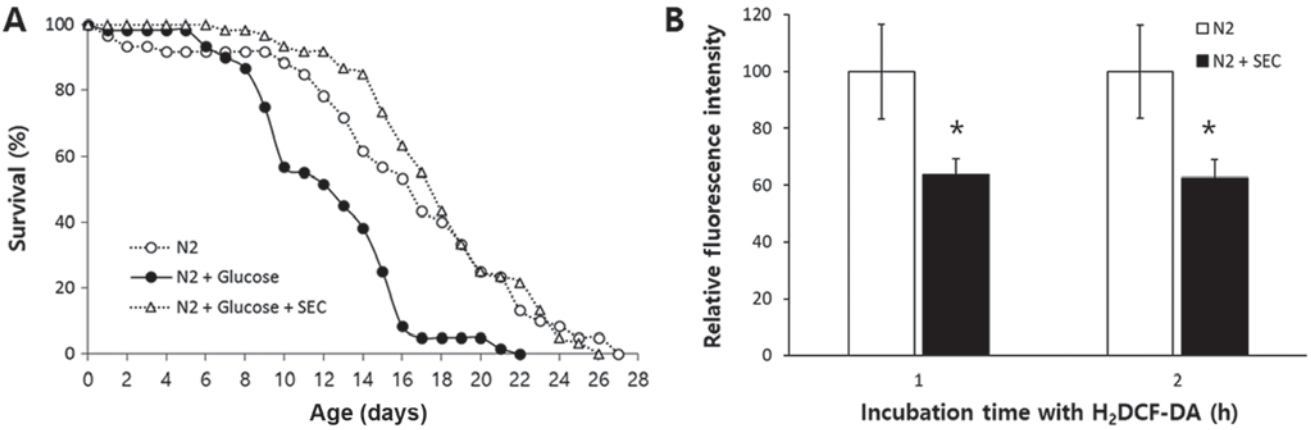

Figure 5. Effect of selenocysteine on high-glucose-induced toxicity and cellular ROS. (A) Effect of high-glucose-diet with or without selenocysteine on the survival of worms was examined (n=60). Decreased survival caused by high-glucose-induced toxicity was recovered by supplementation with selenocysteine. The x- and y-axes indicate the age of the animals and the percentage of survival, respectively. The experiment was repeated three times. (B) ROS levels were measured *in vivo* using a fluorescence multi-reader (n=20). Selenocysteine significantly reduced cellular ROS levels, which has been reported to increase with the high-glucose-diet. Data is presented as the mean ± standard error of the mean (n=20 individual worms). *P<0.05 vs. N2. SEC, 5 mM selenocysteine; ROS, reactive oxygen species; H_2DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate.

DR. Our results provide strong evidence for the development of DR mimetics using selenocysteine. Further studies focusing on the effect of selenocysteine on age-related diseases such as AD, Parkinson's disease, and diabetes mellitus in mammalian disease models and the molecular basis of the effect of selenocysteine are necessary for the understanding of *in vivo* activity of selenocysteine.

The significance of this study is the identification of a novel DR mimetics. Further studies focusing on the cellular pathways involved and the longevity phenotype in other model organisms should be followed in a near future to compare the effect of selenocysteine with other previously reported DR mimetics, such as metformin and resveratrol. So far, many DR mimetics has been reported in many model organisms. Each DR mimetic has both positive and not significant effect on lifespan and healthspan. For example, the effect of metformin on lifespan is not universal (no effect on lifespan of rats) and sex-dependent in fruit fly (61). A recent meta-analysis studies reports no effect of metformin on over-all mortality (62). The effect of resveratrol on lifespan extension was not observed in invertebrate models (63). Therefore, it is important to understand the mechanisms involved with each DR mimetics and to propose a possible combination of

DR mimetics that are effective on multiple species. It is unlikely to find perfect and safe DR mimetics in the near future since the exact mechanism of DR itself is still illusive. However, due to universality of DR effect and difficulty in implementing DR, researches focusing on the identification of novel DR mimetics and elucidation of underlying mechanisms of DR mimetics are invaluable to human health. They are drawing increasing attention in the field of aging.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SHK performed all of the experiments in the present study and wrote the first manuscript. BKK analyzed the effect of selenocysteine on mutants and the DR effect. SKP designed the experiments, and reviewed and edited the final version of the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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